Vitis 45 (3), 131–139 (2006)

Inhibition of crown gall induction by *Agrobacterium vitis* strain F2/5 in grapevine and *Ricinus*

S. Zäuner[1)], J. E. Creasap[2)], T. J. Burr[2)] and C. I. Ullrich[1), 3)]

[1)] Institut für Botanik, Darmstadt University of Technology, Darmstadt, Germany
[2)] Department of Plant Pathology, Cornell University, NYASES, Geneva, USA
[3)] Institute for Biological Control, Federal Biological Centre of Agriculture and Forestry, Darmstadt, Germany

Summary

Biological control measures to prevent or reduce *Agrobacterium vitis*-caused losses in grapevine cultures are a worldwide increasing challenge. In the present study, tumour development in grapevine (*Vitis vinifera* L.) was induced in the sensitive cv. Kerner by infection with *Agrobacterium vitis* strain K306, carrying the p35S*gus*-int plasmid with the *gus* gene as marker for transformation by the wild-type T-DNA. Pre-inoculation with the non-tumorigenic *A. vitis* strain F2/5 prevented tumour induction by K306(p35*gus*-int). Strain M1154, a Tn5 mutant of F2/5 in the *luxR*-like *aviR* gene, partially reduced the biocontrol efficiency compared to the wild-type F2/5. GUS-labelling by K306*gus* was poor in grapevine in contrast to *A. tumefaciens* 281(p35*gus*-int)-induced tumours in *Arabidopsis*, indicating plant species-dependent variable *gus* expression. To use the more reliable direct mRNA expression assay by RT-PCR, a new experimental plant/*A. vitis* system was established with *Ricinus communis* as model plant. *Ricinus*/*A. vitis* galls were available within one week after K306*gus* inoculation, reached diameters up to 5 cm, and contained more abundant GUS staining. An additional transformation marker, mRNA expression of the T-DNA-located *iaaM* oncogene, coding auxin synthesis, was apparent only in tumours induced by the wild-type *A. vitis* strain K306 in the absence of the *gus* construct, which is under the control of the strong 35S CaMV promoter. F2/5 pre-inoculation suppressed GUS staining and *gus* mRNA expression. DAPI staining revealed the loss of vital fluorescent cell nuclei in F2/5-inoculated grapevine tissue and thus inhibition of any successful T-DNA transfer into host cell nuclei. Differentiation of typical circular vessels in globular vascular bundles in M1154-pretreated galls suggests interference with plant auxin metabolism. In conclusion, together with successfully establishing a new experimental model system, *Ricinus*/*A. vitis*, pre-treatment of host tissue with the non-pathogenic strain F2/5 resulted in preventing the integration and expression of the oncogenic T-DNA of *A. vitis* strains by locally necrotizing host cell nuclei.

K e y w o r d s : Biocontrol of *Agrobacterium vitis*, *gus* expression, *Ricinus* model system, RT-PCR, T-DNA expression

Introduction

Crown gall disease in grapevine by *Agrobacterium vitis* is initiated in the same manner as that by *Agrobacterium tumefaciens* (Weiler and Schröder 1987), *i.e.* by transfer and integration of the T-DNA of the bacterial Ti plasmid via wounding of grapevine. *A. vitis* T-DNA encodes the oncogenes *iaaM*, *iaaH* for auxin synthesis, and *ipt* for cytokinin synthesis, in addition to opines, which have been characterized previously (Ophel and Kerr 1990, Otten *et al.* 1996, Momol *et al.* 1998, Burr and Otten 1999). These main genes cause tumour proliferation. Crown gall development retards growth and may lead to complete grapevine decline in most cultivars (Stover *et al.* 1997, Burr and Otten 1999, Otten and Burr 2006). With the exception of planting clean grapevines, reducing injuries, and eradication there are currently no successful control measures that are comparable to those against *A. tumefaciens,* such as the pre-treatment with *A. rhizogenes* strains K84 and 1026. These strains control crown gall development with the production of the specific bacteriocin agrocin (Kerr and Htay 1974, Farrand 1990), however, this agrocin is not effective against *A. vitis* strains (Burr and Otten 1999). Therefore, the search for a successful control of crown gall in grapevine has become important to various grape-growing regions worldwide (Burr and Reid 1994, Bazzi *et al.* 1999, Burr and Otten 1999, Otten and Burr 2006). *A. vitis* strain F2/5, isolated by Staphorst *et al.* (1985), is a promising candidate for grapevine protection. The non-tumorigenic strain F2/5 induces necrosis in green grapevine shoots, roots, and, as recently determined, in cambium (Creasap *et al.* 2005). F2/5 prevents further crown gall development from tumorigenic *A. vitis* strains (Burr and Reid 1994). Unlike *A. rhizogenes* K84 or K1026, control of tumour development with the F2/5 strain in grapevine does not appear to be due to antibiotic production or to competition for attachment to the host cell (Burr *et al.* 1997). In contrast to *A. tumefaciens*, most *A. vitis* strains, including F2/5, induce a hypersensitive response (HR) in tobacco (Zheng *et al.* 2003, Hao *et al.* 2005).

The aim of the present study was to further elucidate the biological control mechanism of the non-pathogenic *A. vitis* strain F2/5 against pathogenic *A. vitis* strains. In particular, the interaction of F2/5 with the host tissue and its effect on the tumour-inducing T-DNA transfer and expression of the *gus*-labelled T-DNA of *A. vitis* strain K306 was

Correspondence to: Dr. C. I. Ullrich, Institute for Biological Control, Federal Biological Centre of Agriculture and Forestry, Heinrichstr. 243, 64287 Darmstadt, Germany. Fax: +49-6151-423-977; E-mail: uleb@bio.tu-darmstadt.de

investigated in woody stems of the susceptible grapevine cv. Kerner. Since tumors develop slowly, though persistently, on woody grapevine stems, and because such woody tissue is a difficult material for molecular biological investigations (BOURQUIN *et al.* 1995), a model system was developed with *Ricinus communis* as host plant for providing transformed tissue more rapidly, and allowing for efficient extraction of nucleic acids.

Material and Methods

P l a n t s : Cuttings (about 1,000) of woody and mature one-year-old shoots of the susceptible grapevine cv. Kerner (*V. vinifera*) were collected in early February in the Palatinate grapevine-growing region (Weingut Glaser, Hainfeld, Germany). Kerner proved to be more susceptible to *A. vitis* infection than the previously used cvs Riesling and Chardonnay (CREASAP *et al.* 2005). The cuttings were surface sterilized with 0.15 % NaOCl and stored at 4 °C until use. The three-node-cuttings were rooted in perlite and transferred to a mixture of perlite : potting soil (LD80) (1:4) and kept in a growth room with 15 h light at 22 °C and 9 h dark at 16 °C until the first leaves became visible. Photon flux density was 145 µmol m^{-2} s^{-1} at plant level. Following the protocol of CREASAP *et al.* (2005), an electric drill was used to bore three holes (2.8 mm diameter drill bit) in woody grapevine cuttings at 1.5 cm below the uppermost actively growing shoot. Each hole was inoculated with 50 µl bacterial suspension, protected with Parafilm and kept for crown gall development up to 12 months under the same conditions in the growth room. Ten cuttings of each treatment were inoculated; tumours were harvested after 2, 3 and 4 months; the experiments were repeated at least 5 times.

Seeds from *Ricinus communis* L. var *gibsonii* cv. Carmencita (Walz Samen, Stuttgart, Germany, approximately 120 plants) were germinated and kept in a growth room with 16 h light at 28 °C and 8 h dark at 22 °C. One-week-old seedlings were wounded with razor blades 1 cm below the cotyledons by 2 cuts of 5 mm length and inoculated with the bacterial suspension, according to ALONI *et al.* (1995). Plants with developing tumours were kept under the same conditions up to 4 months.

B a c t e r i a l s t r a i n s : *A. vitis* strains were grown on potato dextrose agar (Merck, Darmstadt, Germany) ± kanamycin (50 µg ml^{-1}) at 28 °C. Bacteria, as described by CREASAP *et al.* (2005), were obtained from the T. Burr lab (Cornell Univ., Geneva, USA). The tumorigenic octopine strain K306 was used for tumour development and biological control assays. Strain K306(p35S*gus*-int) was generated by conjugation of strain K306 with *Escherichia coli* strain DH5 (Stover *et al.* 1996), carrying the p35S*gus*-int with the *gus* (β-glucuronidase) gene and kanamycin resistance (VANCANNEYT *et al.* 1990). The intron ensures the expression of the *gus* gene only in eukaryotic cells. During infection of plant cells, K306 may transfer T-DNA regions from pTi306, from p35S*gus*-int, or from both. For biocontrol activity the non-tumorigenic but necrosis- and HR-inducing strain F2/5 (ZHENG *et al.* 2003, HAO *et al.* 2005) was used. This F2/5 strain harbours plasmid-encoded genes for tartrate and octopine catabolism (SZEGEDI *et al.* 1999). To assess genes involved in biocontrol activity, a Tn5-generated mutant strain of F2/5, M1154, was also used (HERLACHE *et al.* 2001). In this mutant, the Tn5 transposon, carrying kanamycin resistance, is inserted in the *luxR* homologue, *aviR* (ZHENG *et al.* 2003); it does not induce necrosis in grapevine or HR on tobacco (HERLACHE *et al.* 2001). Prior to inoculation bacteria were grown in liquid potato dextrose broth overnight, and the concentration was adjusted to 10^8 cfu ml^{-1} (optical density at 600 nm = 0.1). The efficiency of F2/5 on crown gall development was tested by inoculating wounds with the same cell density of an F2/5 suspension, or with sterile dist. H_2O as control, 24 h prior to inoculation with K306.

The nopaline wild-type strain *Agrobacterium tumefaciens* C58 was obtained from Dr. Sz. Koncz, Max-Planck-Institut Köln, Germany, and the supervirulent strain *A. tumefaciens* A281 (p35S*gus*-int) carrying an intron-containing β-glucuronidase (GUS) gene under the control of the CaMV 35S-promoter and genes for kanamycin and rifampicin resistance was obtained from the DLO-Centre for Plant Breeding and Reproduction Research (Wageningen, The Netherlands); it has been described by VANCANNEYT *et al.* (1990) and VAN WORDRAGEN *et al.* (1992). These strains were grown in yeast extract broth (YEB) as described previously (PRADEL *et al.* 1996, REZMER *et al.* 1999).

H i s t o c h e m i c a l β - g l u c u r o n i d a s e (G U S) s t a i n i n g : Tumours of transformed plants were cut into 150 µm thick sections with a vibratome (TPI-1000, Polysciences, Eppelheim, Germany). The tissue sections were gently vacuum-infiltrated for 30 min with the incubation solution containing 1 mM 5-bromo-4-chloro-indolyl-β-D-glucuronide (X-Gluc; Molecular probes, Eugene, OR, USA) at pH 7.0 and 0.1 % Tween 20, and were incubated at 37 °C for 1.5 h according to JEFFERSON *et al.* (1987). After histochemical staining for GUS activity the tissue was vacuum-infiltrated for at least 4 h with a clearing solution of 100 % chloral hydrate (w/v)/90 % lactic acid (2:1, v/v). Sections were viewed in 90 % lactic acid with an Aristoplan microscope (Leica, Bensheim, Germany).

Alternatively, for isolation of mRNA from GUS-labelled tumour sections the X-gluc incubation buffer contained RNase inhibitor (Stratagene) to prevent RNA degradation. Immediately after the GUS-staining assay, the sections were stored on ice, and blue stained were separated from unstained tissues with microscope Aesculap holders equipped with razor blade pieces (Aesculap, Tuttlingen, Germany) under a M400 dissection microscope (Wild) according to REZMER *et al.* (1999). For extraction of total RNA, 100 mg of tissue, immediately frozen in liquid nitrogen, were homogenized with a micro-dismembrator (Braun, Melsungen, Germany).

D A P I : For the detection of nuclei, tissue sections were stained with 1 mg ml^{-1} $H_2O_{dist.}$ 4',6-diamidino-2-phenylindol (DAPI) for 10 - 15 min, rinsed twice with 0.1 x PBS (phosphate buffered saline) and viewed under UV light (filter block A, Aristoplan epifluorescence micro-

scope, Leica). Micrographs were reproduced from colour slides taken with the attached Orthomat E camera system (Leica) on Agfachrome CT 100 or 200 ASA daylight film.

Extraction of RNA: For RNA extraction, samples (50 - 200 mg fresh wt) from tumours and control tissues were collected, immediately frozen in liquid nitrogen and homogenized with the micro-dismembrator in Teflon capsules and tungsten carbide balls. Total RNA from *Vitis* was extracted according to DONG and DUNSTAN (1996) and from *Ricinus* using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Synthesis of cDNA and RT-PCR: RT-PCR was performed with the Omniscript Reverse Transcriptase Kit (Qiagen) or the OneStep RT-PCR Kit (Qiagen) as described by the manufacturer. Following the protocols as previously reported by REZMER *et al.* (1999), ALONI *et al.* (2003), and SCHWALM *et al.* (2003), for first-strand cDNA synthesis the optical density (OD) of the nucleic acids from the tissue extracts was measured and 1 µg of nucleic acids were consistently used for the reverse transcription (RT) reaction. A *gus* fragment of the cDNA of *A. vitis* was obtained by PCR using the following coding sequence as primers (Jefferson, R. A., patent number GB 2197653-A 1, 25-May-1988): *gus*3a left (sense) primer was 5'-AACGTCTGGTATCAGCG-3', the right (antisense) primer was 5'-AGGGTAATGCGAGGTAGC-3', resulting in a 801 bp amplificate without the intron, 990 bp with the intron. For tryptophan monooxygenase (*iaaM*; KLEE *et al.* 1984) the left primer (sense) was 5'-TGGTGGATCTGACAATGG-3' and the right primer (antisense) was 5'-GAGCAAGTCGATTGTTGG-3', resulting in a 584 bp amplificate. For the house-keeping plant gene, the cytosolic glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, SHIH *et al.* 1991) the left primer (sense) was 5'-GAATCAACGGATTCGGAAGA-3' and the right primer (antisense) was 5'-AACAACCTTCTTGGCACCAC-3', resulting in a 347-bp amplificate. The amplification was well within the linear range (ALONI *et al.* 2003), so all RT-PCR reactions were run for 35 cycles (PTC 200 multicycler, Biozym, Oldendorf, Germany) as follows: *gus* at 95 °C (60 s), 48 °C (30 s) 72 °C (180 s); *iaaM* at 95 °C (30 s), 48 °C (30 s) 72 °C (60 s); *GAPDH* at 95 °C (30 s), 52 °C (30 s), 72 °C (60 s). RT-PCR fragments were separated on 2 % agarose gels.

Results

Development of *A. vitis*-induced crown galls in grapevine: *A. vitis* strain K306 induces galls of considerable size up to 2.5 cm diameter within 3 months at stems of *V. vinifera* cv. Kerner (Fig. 1 a). As test for tissue transformation by *A. vitis* T-DNA, tumour tissue sections were GUS stained.

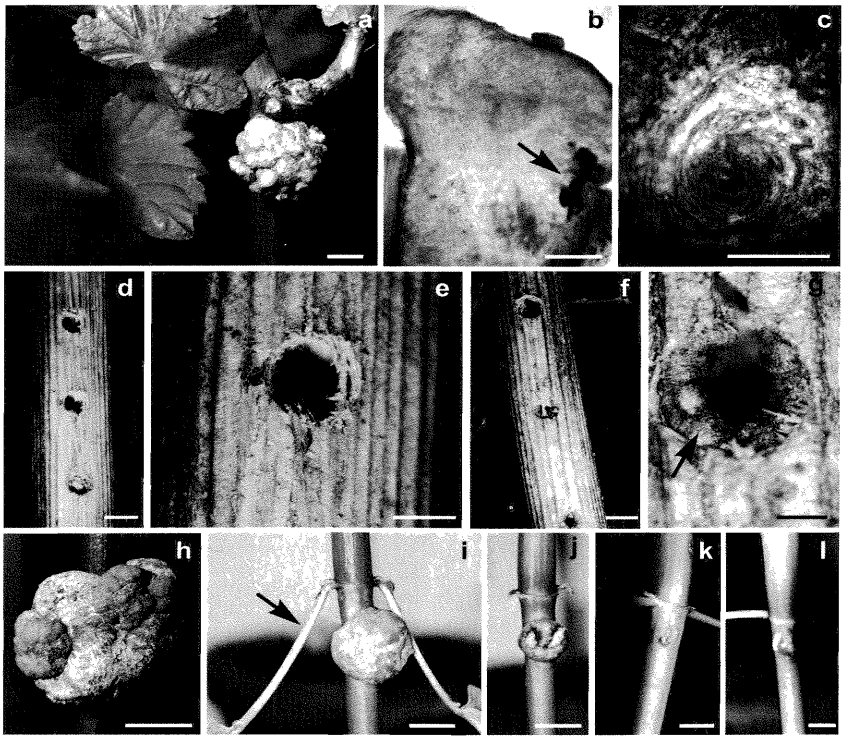


Fig. 1: *Agrobacterium vitis*-induced crown gall development in grapevine (**a-g**) and *Ricinus* (**h-l**). **a**: 3-month-old crown gall tumours on the grapevine variety Kerner induced by inoculation with *A. vitis* K306. **b**: Tumour section, GUS-stained (arrow). **c**:Prominent circular vessels in globular bundles in M1154/K306-induced tumours (toluidine blue staining). **d**, **e**: Grapevine stems inoculated with *A. vitis* strains F2/5 and K306; within 3 months *post inoculationem* (pi) no callus and no tumour development; overview and detail. **f**, **g**: Grapevine stems inoculated with *A. vitis* strains M1154 and K306; at 3 months pi small calli at the margin of the wound developed (arrow); overview and detail. **h-l**: Tumors induced in *Ricinus communis* by **h**, *A. vitis*; 4-month-old; **i**: *A. tumefaciens* strain C58, causing epinastic reaction of cotyledons (arrow). **j**: *A. vitis* strain K306, with rapid abscission of cotyledons. **k**: *A. vitis* strains F2/5 and K306, no epinasty of cotyledons. **l**: *A. vitis* strains M1154 and K306, with slight epinasty; **i-l**: 3 week-old tumours. Bars = 1 mm (**b**, **c**, **g**), 2 mm (**e**), 4 mm (**d**, **f**), 1 cm (**a**), 1.5 cm (**h-l**).

Very few areas were labelled (Fig. 1 b, 4 h), inferring low transformation rate in spite of vigorous tumour proliferation. Therefore, in addition mRNA analysis by RT-PCR was used as direct method for estimating the efficiency of transformation. In 4-month-old tumours induced by strain K306*gus* on *Vitis* stems, *gus* mRNA expression (*i.e.* successful transformation) was detected as an 801 bp amplificate (Fig. 2). The amplified *gus* DNA, with a length of 990 bp due to the intron in the bacterial plasmid, appeared only in bacterial control samples (Fig. 2, lane *gus Av*) but not in the tumour tissue (Fig. 2, lane T2), thus excluding bacterial contamination of the examined tumour tissue.

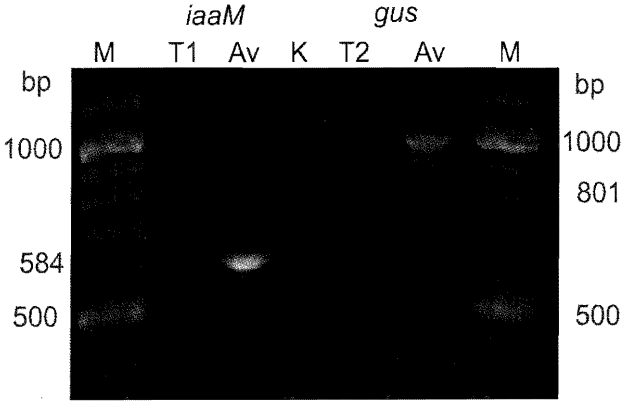


Fig. 2: mRNA expression of *gus* and *iaaM* in 4-month-old tumors of grapevine. (**T1**) no *iaaM* expression in tumour tissue. (**Av**) *iaaM* expression in positive control only in samples of *A. vitis* bacteria. (**K**) uninoculated tissue (no tumours), no expression of *iaaM* or *gus*. (**T2**) Expression of *gus* from tumour tissue with an 801 bp amplificate. (**Av**) The amplified DNA with a length of 990 bp (contains the intron) appeared only in mere bacterial control samples (*A. vitis gus*), not in the tumour tissue.

Though the transferred T-DNA contained the *iaaM* gene for additional auxin synthesis, it could not be detected in the presence of the *gus* marker gene, probably due to its strong 35S promoter (Fig. 2, lane T1). As control for correct primer design and further experimental procedures, samples of bacteria alone showed a distinct amplificate of the *iaaM* gene (Fig 2, lane *iaaM Av*). In the non-infected control tissue no T-DNA gene expression was present (Fig. 2, lane K).

Pre-inoculation of wounds with the non-pathogenic *A. vitis* strain F2/5 prevented tumour development by *A. vitis* strain K306 (Fig. 1 d,e), whereas the Tn5-induced mutant strain M1154 did not completely inhibit callus induction, but retarded further tumour proliferation (Fig. 1 f, g). Circular vessels of globular bundles were characteristic within this tissue, showing impaired tumour growth (Fig. 1 c) and indicating high, but disturbed, basipetal flux of auxin.

Development of *A. vitis*-induced crown galls in *Ricinus*: Crown gall growth in mature woody stems of *Vitis* is slow and mRNA and DNA extraction is problematic due to the large portion of lignified fibres and vessels in relation to living cambium and parenchyma cells. The concentration of nucleic acid degrading polyphenols of this woody tissue is high, making nucleic acid isolation difficult. Although *A. vitis* is commonly found in field-grown grapevines, it induces tumours on a few other test plant species such as *Lycopersicon esculentum* and *Kalanchoë tubiflora* as well (BURR and OTTEN 1999). In previous studies, *Ricinus* proved to be an excellent experimental model plant to analyse physiological, biochemical, and molecular characteristics of *Agrobacterium tumefaciens*-induced tumour development (ULLRICH and ALONI 2000, ALONI and ULLRICH 2006). Indeed, on young *Ricinus* stems *A. vitis* induced rapidly proliferating tumours (Fig. 1 h) that were slightly smaller than those incited by *A. tumefaciens* strain C58 at comparable ages (Figs 1 i *vs.* j and 3; here, three weeks *post inoculationem*, pi).

Pre-treatment of wounds with strain F2/5 prevented tumour development by the subsequently applied tumorigenic strain K306 (Figs 1 k and 3). As in grapevine, the Tn5 mutated strain M1154 inhibited tumour development considerably, but not completely (Figs 1 l and 3). Because *Ricinus* reacted to inoculation with the pathogenic and non-pathogenic *A. vitis* strains in the same way as grapevine, the results allowed us to use *Ricinus* as an experimental model plant for RT-PCR analysis of specific *A. vitis* T-DNA mRNA expression, which is important for the evaluation of the efficiency of the biological control mechanisms of strain F2/5.

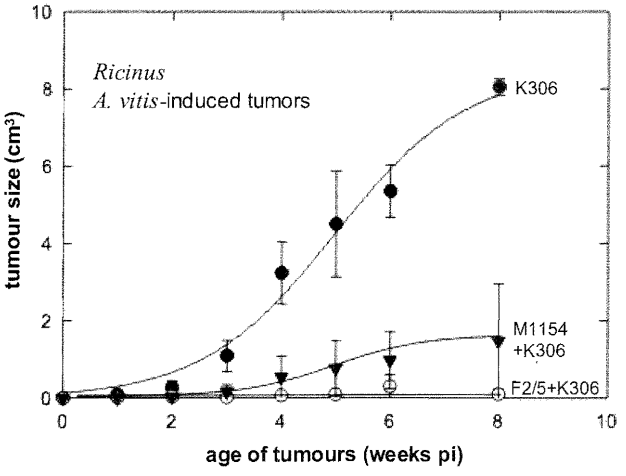


Fig. 3: Time course of *A. vitis*-induced crown gall growth in *Ricinus*; inoculation with strain K306*gus* (closed circles); strains F2/5 and K306*gus* (open circles) and with strains M1154 and K306*gus* (closed triangles); mean values ± SE (n = 5 to 32).

The assay on the transformation rate of *Ricinus* by strain K306*gus*, using the indirect method of histochemical GUS staining of tumour sections, revealed, in comparison with grapevine, more abundant but still scattered blue patches, although the whole tumour should be transformed by the T-DNA of *A. vitis* (Fig. 4 a).

gus mRNA expression detected by RT-PCR in K306-induced tumours was apparent within one week pi and up to 5 weeks pi with an amplificate of 801 bp, as expected (Fig. 5 a). Samples of uninoculated tissue (Fig. 5 a, lane Pr) did not have *gus* expression.

Pre-inoculation of the wounds with strain F2/5 prevented potential *gus* expression in cells subsequently inoculated with the tumorigenic strain K306*gus*. These cells

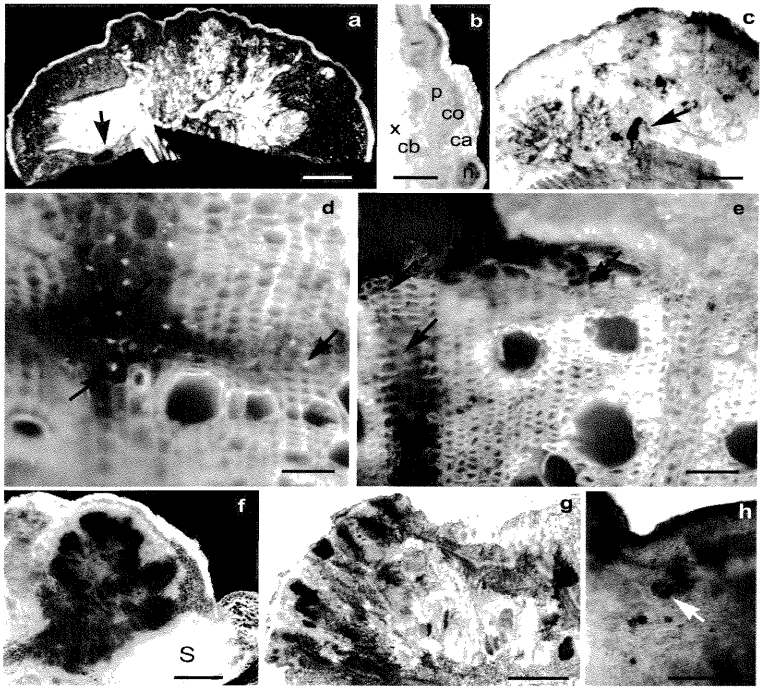
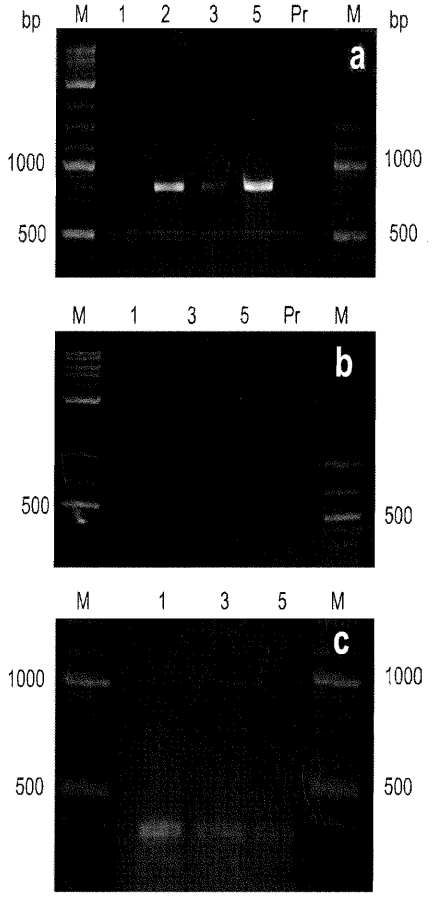


Fig. 4: T-DNA *gus* expression in 6-week-old tumours of different host plant species. **a**: *Ricinus* inoculated with *A. vitis* strain K306 (*gus*-int), with GUS-patches (blue). **b**: *Ricinus* inoculated with *A. vitis* strains F2/5 and K306, no tumour development and no GUS labelling (x, xylem, cb, cambium, p, phloem, co, cortex, ca, callus, n, necrosis). **c**: *Ricinus* inoculated with *A. vitis* strains M1156 and K306*gus* with minor GUS labelling. **d**, **e**: DAPI staining of cell nuclei in grapevine tissue sections. **d**: Control: uninoculated tissue with distinct fluorescent nuclei in rays of phloem, cambium and xylem (arrows), and **e**: inoculated with F2/5 and K306 strains no DAPI fluorescence in nuclei of the cambium (the site of infection) and xylem rays (arrows). **f**: *Arabidopsis thaliana* inoculated with *A. tumefaciens* strain A281 p35S(*gus*-int) is abundantly GUS labelled; S, shoot rosette with leaf basis. **g**: *Ricinus* inoculated with *A. tumefaciens* strain A281 p35S(*gus*-int) with GUS-labelled patches. **h**: Grapevine inoculated with strain K306 with poor GUS labelling (arrow). Bars = 100 µm (**d**, **e**), 500 µm (**f**), 1mm (**h**), 2 mm (**g**), 3 mm (**a**-**c**).



lacked the indigo colouring (GUS-staining) typical of GUS assays (Fig. 4 b), and *gus* mRNA was absent from these cells as indicated in RT-PCR (Fig. 5 b). The expression of the house-keeping gene *GAPDH* of the host tissue as control for equal loading of the gels was present in all samples used for analysis of F2/5 plus K306*gus* inoculated tissue (Fig 5 c).

Sections of small *Ricinus* tumours induced by M1154 and K306(p35S*gus*-int) (Fig. 1 l) contained GUS-stained areas (Fig. 4 c), confirming that the mutated gene appears partially responsible for biocontrol activity, in addition to necrosis in grapevine shoot explants and HR induction on tobacco.

To determine the extent of transformation throughout the *A. vitis*-induced tumour, GUS-stained (blue) and unstained areas of tissue sections (Fig. 4 a) were carefully separated by microdissection and examined by RT-PCR for

Fig. 5, **a**: Time course of *gus* mRNA from *A. vitis*-induced tumours of *Ricinus*. Numbers at the top denote weeks *post inoculationem*. (Pr) negative control samples of uninoculated tissue. *gus* expression amplificates in tumours with 801 bp; no bands at 990 bp (fragment plus intron), excluding bacterial contamination. **b**: Time course without expression of the *gus* gene in *Ricinus*, inoculated with *A. vitis* strains F2/5 and K306, indicating absence of transcription of the *gus* gene. (Pr) Negative control sample with non-infected tissue. **c**: Time course of the expression of the house-keeping gene *GAPDH* in F2/5 and K306 inoculated *Ricinus* tumour tissue.

gus mRNA expression. Only in GUS (blue)-stained tissue *gus* mRNA expression was found; unstained tissue did not have any *gus* mRNA expression (Fig. 6 a, lanes u *vs.* s).

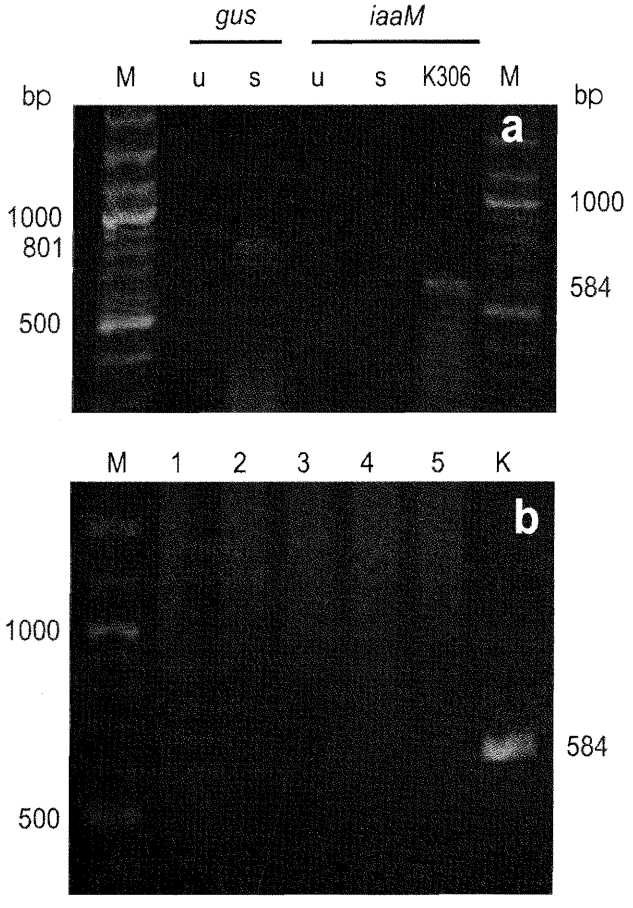


Fig. 6, **a**: Tissue-specific expression of *gus* and *iaaM* genes in 6-week-old *Ricinus* tumors. Absence of *gus* expression in unstained (u) but presence of *gus* expression in blue stained (s) tumour sections. No *iaaM* mRNA expression in K306*gus*-induced tumours. In lane K306 mRNA expression of *iaaM* in 2-week-old crown galls induced by K306 strain (without the *gus*-fusion). **b**: Time course of lack of expression of the *iaaM* gene in *Ricinus* tumours after inoculation with *A. vitis* strain K306. (K) Positive control samples with K306 bacteria only. Numbers on the top indicate weeks *post inoculationem*.

Though the T-DNA is transferred to the host tissue, and tumours grow due to additional auxin production upon expression of the *iaaM* and *iaaH* genes, *iaaM* mRNA was not expressed in unstained (u) or in blue-stained (s) tissue (Fig. 6 a). In analyses of tumours induced by the K306 strain lacking the *gus* reporter gene and its 35S promoter, however, distinct *iaaM* mRNA was expressed with the expected amplificate of 584 bp (Fig. 6 a, lane K306). To exclude possible time-dependent variations in the *iaaM* expression pattern, the time course of 1 - 5-week-old tumour samples was analysed. In all samples *iaaM* expression was suppressed when the galls had been induced by the K306 strain with the *gus* gene and 35S promoter (Fig. 6 b), whereas in bacterial control samples (K) an amplificate at 584 bp was detectable (Fig. 6 b).

The lack of *gus* mRNA expression in F2/5-treated wound tissue raised the question as to whether the T-DNA gene expression or the transfer and integration of the T-DNA were inhibited. Therefore, grapevine tissue inoculated with both strains was stained with DAPI to examine the cell nuclei at the sites of inoculation and establish if they were still functional. In uninoculated control tissue, ray cells in the phloem, cambium, and xylem showed distinct fluorescent nuclei upon DAPI staining (Fig. 4 d). Tissue inoculated with F2/5 and K306*gus* contained no fluorescent, hence no intact, nuclei (Fig. 4 e).

Our current results on differing *gus* mRNA expression in GUS-stained and unstained tumour sections indicated a considerable variability of *gus* expression, apparently dependent on the host plant species. Similar GUS patchiness of tumour tissue, reported earlier from *A. tumefaciens*-induced tumours in *Ricinus* (REZMER *et al.* 1999), was found to consist of 100 % transformed parenchyma cells throughout the tumour, as detected by *gus* DNA analysis. To further understand the absence of *gus* expression in unstained tissue in contrast to the blue patches of tumour sections, different plant species were inoculated with the same *Agrobacterium* strain and the same plant species were inoculated with different tumorigenic strains of *Agrobacterium* carrying p35S*gus*-int. *Arabidopsis thaliana* tumours displayed nearly complete T-DNA-*gus* expression, indicating efficient transformation and expression by strain *A. tumefaciens* A281(35S*gus*-int) via rosette inoculation (according to SCHWALM *et al.* 2003) (Fig. 4 f), whereas *Ricinus* stem tumours, induced by the same strain, displayed only scattered blue patches (Fig. 4 g). Inoculation of *Ricinus* with *A. vitis* strain K306*gus* showed a similar pattern with numerous blue patches (Fig. 4a). Inoculation of grapevine with the same strain K306*gus* resulted in large tumour parenchyma with only very poorly blue-stained cell clusters (Figs 1 b, 4 h).

Discussion

Biological control of bacterial plant diseases is of increasing interest and importance. *Agrobacterium vitis* can cause severe losses in many grapevine cultivars worldwide (BURR and OTTEN 1999, OTTEN and BURR 2006); therefore, mechanisms of biological control are under investigation. One promising possibility is the non-tumorigenic *A. vitis* strain F2/5 (STAPHORST *et al.* 1985, OTTEN and BURR 2006). This strain is characterized by an induction of a black necrosis in grapevine roots and shoots, a necrosis more severe than that induced by other *A. vitis* strains (BURR *et al.* 1987), and hypersensitive response in tobacco (HERLACHE *et al.* 2001). F2/5 also prevents the development of cambial wound callus cells that are susceptible to T-DNA incorporation and expression (CREASAP *et al.* 2005). It was assumed that the mechanism of necrosis production was related to the ability of *A. vitis* to cause an HR-like response in non-host plants (HERLACHE *et al.* 2001) and the polygalacturonase activity of *A. vitis* in general (RODRIGUEZ-PALENZUELA *et al.* 1991). The genes responsible for HR, and possibly biocontrol, have been identified as the *luxR* homologues, *aviR* and *avhR*, which constitute a complex of multiple gene expression (ZHENG *et al.* 2003, HAO *et al.* 2005, OTTEN

and BURR 2006). On the host plant level, it was unknown as to whether the biocontrol effect of strain F2/5 was due to an inhibition of T-DNA expression from tumorigenic strains, or whether F2/5 inhibited the transfer and integration of the oncogenic T-DNA into host plant tissue.

As previously reported by CREASAP *et al.* (2005) pre-inoculation of grapevine stems with strain F2/5 prevented GUS-staining of the host tissue, which was confirmed in this study, with the additional result that F2/5 prevents T-DNA gene expression and subsequent tumour development (Fig. 1 d, e). Histochemical GUS staining as an indirect test, however, was not completely reliable and was dependent upon the host plant species under investigation. In *Arabidopsis thaliana* up to 80 % of the tumour tissue was GUS-stained after inoculation with the supervirulent *Agrobacterium tumefaciens* strain A281 p35S(*gus*-int) (Fig. 4 f), whereas the same strain induced only scattered GUS patches in *Ricinus communis* (Fig. 4 g), although large tumours developed. The *A. vitis* strain K306, which carries on a second plasmid the same construct of 35S(*gus*-int), induced similarly scattered blue patches in *Ricinus* (Fig. 4 a) whereas grapevine was only poorly labelled (Figs 1 b, 4 h). These results indicate that the content of polyphenols and oxidizing enzymes, such as peroxidase, which increases with increasing lignification, is highest in woody grapevine stems, there reducing GUS expression.

Consequently, to evaluate the transformation of host tissues mRNA expression studies via RT-PCR were performed. In tumours from grapevine tissue, mRNA of the *gus* marker gene from *A. vitis* strain K306 could be detected (Fig. 2, lane T2). In samples containing bacteria alone, DNA from the *gus* gene, including the intron, was detected with the expected amplificate of 900 bp (Fig. 2, lane *Av*); however, this amplificate was not detected in the tumour tissue (lane T2), thus excluding bacterial contamination in tumour tissue. As an alternative marker for transformation, *iaaM* (auxin) expression was examined, but it could only be detected in samples of bacteria alone (Fig. 2, lane *Av*), not in grapevine (Fig. 2, lane T1). Grapevine is a difficult experimental tissue for mRNA analyses due to the slow growth of tumours, its high content of polyphenols (BOURQUIN *et al.* 1995), and the low portion of parenchymatous cells in the woody tissue. Because *Ricinus* proved to be an excellent model system to study tumour development induced by *A. tumefaciens* (ULLRICH and ALONI 2000, ALONI and ULLRICH 2006), we also tested the response of *Ricinus* to infection by *A. vitis*, although it was reported that *A. vitis* causes necrosis only in grapevine roots and HR in non-host plants (HERLACHE *et al.* 2001). *A. vitis* induced rapidly growing tumours in *Ricinus* with a diameter of about 5 cm within 4 months (Figs 1 h, j, 3). In *Ricinus* F2/5 similarly prevented crown gall development as in grapevine (Figs 1 k, 3). Thus, *Ricinus* turned out to be a very useful model plant for further analysis of *A. vitis* pathogenicity and its biological control.

Within one week pi the *gus* mRNA of *A. vitis* was detectable in small *Ricinus* galls (Fig. 5 a). Corresponding to the visible prevention of tumour proliferation by strain F2/5 (Figs. 1 k, 3), *gus* mRNA expression was not apparent throughout 5 weeks pi (Fig. 5 b), as examined in the few developing callus cells and in accordance with the previously reported absence of histochemical GUS staining (Fig. 4 b) (this study and CREASAP *et al.* 2005).

To assess the reliability of the *gus* mRNA expression as an indicator of successful T-DNA integration and expression and biocontrol by strain F2/5, the *gus* mRNA expression was found to be present only in blue GUS-stained, not in unstained tissue areas (Fig. 6 a, u *vs.* s). This corresponds to earlier findings that *gus* mRNA was detectable only in 25 % of the cells of the living tumour tissue; however, *gus* DNA was present in 100 % of the parenchymatic cells (REZMER *et al.* 1999). Therefore, *iaaM* mRNA as a potential marker of successful T-DNA expression was again examined. In galls induced by K306(p35S*gus*-int), *iaaM* was not expressed (Fig. 6 a) at any time during tumour development (Fig. 6 b), only in bacterial cell samples (Fig. 6 b, lane K). Subsequently, we examined tumours induced by K306 without p35S*gus*-int. In tissue from these tumours, *iaaM* was easily detectable in developing *Ricinus* galls (Fig. 6 a). A possible cause for this problem may be the inhibition of expression of T-DNA genes, which are not under control of the strong 35S CaMV promoter. Our results correspond to the previous report that strong promoters like the 35S CaMV of the *gus* gene in our constructs inhibit the synthesis of auxin and cytokinins in grapevine crown galls (TINLAND *et al.* 1991). Additionally, NAGATA *et al.* (1987) reported that the chloramphenicol acetyltransferase (CAT), also under the 35S CaMV control, is strongly expressed only in the S-phase of growth, further indicating the strength of this promoter which suppresses the expression of non-target genes.

The F2/5 mutant, M1154, contains the Tn5 transposon in the *luxR*-like *aviR* gene and is affected in biological control ability against tumorigenic strains. Unlike F2/5, M1154 is unable to prevent tumour growth completely, and M1154 allows formation of small tumours (Fig. 1 f, g). The biocontrol activity of strain M1154 was similar in *Ricinus*, where *gus* expression from K306(p35S*gus*-int) was detected (Figs. 1 l, 3, 4 c), indicating that the *aviR* gene may be involved in biological control. Additional research has indicated that another, possibly unrelated gene appears to be involved in biological control. The mutant M852 contains a Tn5 disruption in a *clpA* homologue and was produced in the same study that generated M1154 (HERLACHE *et al.* 2001). Further work is being conducted on this strain to determine the mechanism of biological control on *A. vitis* strain F2/5.

Although the precise means of biocontrol are not yet known, one factor likely involved is F2/5's ability to induce necrosis in woody grapevine tissue. DAPI staining of F2/5 and K306-inoculated grapevine tissue revealed that no nuclei in the cambium and xylem rays were stainable, and, hence, were no longer intact. This indicates that the essential site of biocontrol action is the host tissue, to which T-DNA cannot be transferred, because the cells had been killed by F2/5 pre-treatment.

In the small M1154 and K306-induced galls, auxin metabolism seems to be affected, as evident from the abundant development of spiral and circular vessels in globular bundles (Fig. 1 c). Still functional in local water transport

(SCHURR *et al.* 1996), they are known to differentiate when the basipetal auxin flow and basipetal cellular efflux of auxin are inhibited (SACHS and COHEN 1982, ALONI *et al.* 1995), possibly by PIN-protein dislocations. This suggests that the biocontrol process interferes with the plant phytohormone metabolism as well.

In conclusion, the present results indicate that the nontumorigenic *A. vitis* strain F2/5 not only prevents the expression of the oncogenes, as evidenced by the lack of *gus* expression shown in this study, but also induces a severe necrosis in the host tissue. In the absence of intact nuclei, no T-DNA can be imported and stably integrated into the host cells. Strain F2/5 also appears to interfere with auxin metabolism in the host plant. Additionally, by establishing the *Ricinus/A. vitis* plant/pathogen system, a useful experimental model system was provided for further molecular biological analyses of host plant/pathogen/biocontrol interactions.

Acknowledgements

We thank Drs. K. SCHWALM and C. REZMER for helpful suggestions and JEC gratefully acknowledges an Alfred-Toepfer/Alexander-von-Humboldt student award.

References

ALONI, R.; PRADEL, K. S.; ULLRICH, C. I.; 1995: The three-dimensional structure of vascular tissues in *Agrobacterium tumefaciens*-induced crown galls and in the host stems of *Ricinus communis* L. Planta **196**, 597-605.

ALONI, R.; SCHWALM, K.; LANGHANS, M.; ULLRICH, C. I.; 2003: Gradual shifts in sites of free-auxin-production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis. Planta **216**, 841-853.

ALONI, R; ULLRICH, C. I.; 2006: Biology of crown gall tumors. In: T. Tzfira, V. H. Citovsky (Eds): *Agrobacterium*, from Biology to Biotechnology. Springer, Heidelberg (in press).

BAZZI, C.; ALEXANDROVA, M.; STEFANI, E.; ANACLERIO, F.; BURR, T. J.; 1999: Biological control of *Agrobacterium vitis* using non-tumorigenic agrobacteria. Vitis **38**, 31-35.

BOURQUIN, J. C.; OTTEN, L.; WALTER, B.; 1995: PCR-RFLP analysis of *Vitis*, *Ampelopsis* and *Parthenocissus* and its application to the identification of rootstocks. Vitis **34**, 103-108.

BURR, T. J.; BISHOP, A. L.; KATZ, B. H.; BLANCHARD, L. M.; BAZZI, C.; 1987: A root-specific decay of grapevine caused by *Agrobacterium tumefaciens* and *A. radiobacter* biovar 3. Phytopathology **77**, 1424-1427.

BURR, T; OTTEN, L; 1999: Crown gall of grape: biology and disease management. Annu. Rev. Phytopathol. **37**, 53-80.

BURR, T. J.; REID, C. L.; 1994: Biological control of grape crown gall with nontumorigenic *Agrobacterium vitis* strain F2/5. Am. J. Enol. Vitic. **45**, 2 13-219.

BURR, T. J.; REID, C. L.; TAGLIATI, E.; BAZZI, C.; SÜLE, S.; 1997: Biological control of grape crown gall by strain F2/5 is not associated with agrocin production or competition for attachment sites on grape cells. Phytopathology **87**, 706-711.

CREASAP, J. E.; REID, C. L.; GOFFINET, M. C.; ALONI, R.; ULLRICH, C. I.; BURR, T. J.; 2005: Effect of wound position, auxin and *Agrobacterium vitis* strain F2/5 on wound-healing and crown gall in grapevine. Phytopathology **95**, 362-367.

DONG, J. Z.; DUNSTAN, D. I.; 1996: A reliable method for extraction of RNA from various conifer tissues. Plant Cell Rep. **15**, 516-521.

FARRAND, S. K.; 1990: *Agrobacterium radiobacter* K84: A model biocontrol system. In: R. R. BAKER, P. E. DUNOV (Eds): New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases, 679-691. Alan R. Liss. New York.

HAO, G.; ZHANG, H.; ZHENG, D.; BURR, T. J.; 2005: A *luxR* homolog *avhR* in *Agrobacterium vitis* affects the development of a grape-specific necrosis and a tobacco hypersensitive response. J. Bacteriol. **187**, 185-192.

HERLACHE, T. C.; ZHANG, H.; REID, S.; ZHENG, D.; BASARAN, P.; THAKER, M.; BURR, A.; BURR, T. J.; 2001: Mutations that affect *Agrobacterium vitis*-induced grape necrosis also alter its ability to cause a hypersensitive response on tobacco. Phytopathology **91**, 966-972.

JEFFERSON, R.; KAVANAGH, T.; BEVAN, M; 1987: GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**, 3901-3907.

KERR, A.; HTAY, K.; 1974: Biological control of crown gall through bacteriocin production. Physiol. Plant Pathol. **4**, 37-44.

KLEE, H; MONTOYA, A; HORODYSKI, F.; LICHTENSTEIN, C.; GARFINKEL, D.; FULLER, S.; FLORES, C., PESCHON J, J.; NESTER, E.; GORDON, M.; 1984: Nucleotide sequence of the tms genes of the pTiA6NC octopine Ti plasmid: Two gene products involved in plant tumorigenesis. Proc. Natl. Acad. Sci. USA **81**, 1728-1732.

MOMOL, E. A.; BURR, T. J.; REID, C. L.; MOMOL, M. T.; HSEU, S. H.; OTTEN, L. 1998: Genetic diversity of *Agrobacterium vitis* as determined by DNA fingerprints of the 5'-end of the 23S rRNA gene and random amplified polymorphic DNA. J. Appl. Microbiol. **85**, 685-692.

NAGATA, T.; OKADA, K.; KAWAZU, T.; TAKEBE, I.; 1987: Cauliflower mosaic virus 35S promoter directs S phase specific expression in plant cells. Mol. Gen. Genet. **207**, 242-244.

OPHEL, K.; KERR, A.; 1990: *Agrobacterium vitis* sp. nov., formerly designated as *Agrobacterium* biovar 3 from grapevine. Int. J. Syst. Bacteriol. **40**, 236-241.

OTTEN, L.; DERUFFRAY, P.; MOMOL, E.A.; MOMOL, M. T.; BURR, T. J.; 1996: Phylogenetic relationships between *Agrobacterium vitis* isolates and their Ti plasmids. Mol. Plant-Microbe Interact. **9**, 782-786.

OTTEN, L.; BURR, T. J.; 2006: *Agrobacterium*: A disease-causing bacterium. In: T. TZFIRA, V. H. CITOVSKY (Eds): *Agrobacterium*, from Biology to Biotechnology. Springer, Heidelberg (in press).

PRADEL, K. S.; REZMER, C.; KRAUSGRILL, S.; RAUSCH, T.; ULLRICH, C. I.; 1996: Evidence for symplastic phloem unloading with concomitant high activity of acid cell wall invertase in *Agrobacterium tumefaciens*-induced plant tumors. Bot. Acta **109**, 397-404.

REZMER, C; SCHLICHTING, R.; WÄCHTER, R.; ULLRICH, C. I.; 1999: Identification and localization of transformed cells in *Agrobacterium tumefaciens*-induced plant tumors. Planta **209**, 399-405.

RODRIGUEZ-PALENZUELA, P.; BURR, T. J.; COLLMER, A., 1991: Polygalacturonase is a virulence factor in *Agrobacterium tumefaciens* biovar 3. J. Bacteriol. **175**, 6547-6552.

SACHS, T.; COHEN , D.; 1982: Circular vessels and the control of vascular differentiation in plants. Differentiation **21**, 22-26.

SCHURR, U.; SCHUBERTH, B.; ALONI, R.; PRADEL, K.; SCHMUNDT, D.; JÄHNE, B.; ULLRICH, C. I.; 1996: Structural and functional evidence for xylem-mediated water transport and high transpiration in *Agrobacterium tumefaciens*-induced tumors of *Ricinus communis*. Bot. Acta **109**, 405-411.

SCHWALM, K; ALONI, R.; LANGHANS, M.; HELLER, W.; STICH, S.; ULLRICH, C. I.; 2003: Flavonoid-related regulation of auxin accumulation in *Agrobacterium tumefaciens*-induced plant tumors. Planta **218**, 163-178.

SHIH, M. C.; HEINRICH, P. C.; GOODMAN H. M.; 1991: Cloning and chromosomal mapping of nuclear genes encoding chloroplast and cytosolic glyceraldehyde-3-phosphate dehydrogenase from *Arabidopsis thaliana*. Gene **104**, 133-138.

STAPHORST, J. L.; VAN ZYL, F. G. H.; STRIJDOM, B. W.; GROENEWOLD, Z. E.; 1985: Agrocin-producing pathogenic and nonpathogenic biotype-3 strains of *Agrobacterium tumefaciens* active against biotype-3 pathogens. Curr. Microbiol. **12**, 45-52.

STOVER, E. W.; BURR, T. J.; SWARTZ, H. J.; 1996: Transformation of crown gall resistant and susceptible *Vitis* genotypes by *Agrobacterium vitis*. Vitis **35**, 29-33.

STOVER, E. W.; SWARTZ, H. J.; BURR, T. J.; 1997: Crown gall formation in a diverse collection of *Vitis* genotypes inoculated with *Agrobacterium vitis*. Am. J. Enol. Vitic. **48**, 26-32.

SZEGEDI, E.; SÜLE, S.; BURR, T. J.; 1999: *Agrobacterium vitis* strain F2/5 contains tartrate and octopine utilization plasmids which do not encode functions for tumour inhibition on grapevine. J. Phytopathology **147**, 665-669.

TINLAND, B.; KARES, C.; HERRMANN, A.; OTTEN, L.; 1991: 35S-β-glucuronidase blocks biological effects of cotransferred *iaa* genes. Plant Mol. Biol. 16, 853-864.

ULLRICH, C. I.; ALONI, R.; 2000: Vascularization is a general requirement for growth of plant and animal tumours. J. Exp. Bot. **51**, 1951-1960.

VANCANNEYT, G; SCHMIDT, R; O'CONNOR-SANCHEZ, A; WILLMITZER, L; SANCHEZ-ROSA, M; 1990: Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol. Gen. Genet. **220**, 245-250.

VAN WORDRAGEN, M. F.; DE JONG, J.; SCHORNAGEL, M. J.; DONS, H. J. M. 1992: Rapid screening for host-bacterium interactions in *Agrobacterium*-mediated gene transfer to chrysanthemum, by using the GUS-intron gene. Plant Sci. **81**, 207-214.

WEILER E. W.; SCHRÖDER, J.; 1987: Hormone genes and crown gall disease. Trends Biochem. Sci. **12**, 271-275.

ZHENG, D.; ZHANG, H.; CARLE, S.; HAO, G.; HOLDEN, M. R.; BURR, T. J.; 2003: A *luxR* homolog, *aviR*, in *Agrobacterium vitis* is associated with induction of necrosis on grape and a hypersensitive response on tobacco. Mol. Plant Microbe Interact. **16**, 650-658.

Received March 3, 2006